

# Selective Interaction of Complexin with the Neuronal SNARE Complex

DETERMINATION OF THE BINDING REGIONS\*

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Complexins are evolutionarily conserved proteins that specifically bind to soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes and thus may regulate SNARE function. Using purified proteins, we have performed a detailed analysis of the structure of complexin and of its interaction with SNARE proteins. NMR spectroscopy revealed that isolated complexins have no tertiary structure but contain an unusual  $\alpha$ -helical middle domain of approximately 58 amino acids that overlaps with the most highly conserved region of the molecules. Complexins form a stable stoichiometric complex with the central domain of the ternary SNARE complex, whereas no binding was observed to monomeric SNAREs. Using a combination of limited proteolysis, deletion mutagenesis, and NMR spectroscopy, we found that the helical middle region of complexin is responsible for binding to the SNARE complex. Binding was highly sensitive to substitution of syntaxin 1 or synaptobrevin 2 with other SNARE homologs but less sensitive to substitution of SNAP-25. In addition, a stretch of 12 amino acids in the middle of the SNARE motif of syntaxin 1A was able to confer binding activity to the non-binding relative syntaxin 4. Furthermore, disassembly of ternary complexes is not affected by complexins. We conclude that complexins are specific ligands of the neuronal core complex that bind with a central  $\alpha$ -helical domain, probably to the middle of the surface groove formed by synaptobrevin and syntaxin. Complexins may regulate the function of ternary complexes and control membrane fusion through this interaction.

Intracellular membrane fusion events are mediated by conserved sets of membrane-bound proteins referred to as

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SNAREs<sup>1</sup> (acronym for soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) (1). SNAREs comprise a family of proteins that is distinguished by the presence of the SNARE motif, a homologous stretch of approximately 60 amino acids that is usually localized adjacent to the membrane anchor domains (2–4). Best characterized are SNARE proteins functioning in neuronal exocytosis. They include the synaptic vesicle protein synaptobrevin (also referred to as vesicle-associated membrane protein), and the synaptic plasma membrane proteins SNAP-25 and syntaxin 1. These proteins form a stable ternary complex, the core complex, that can be reversibly disassembled by the ATPase NSF (*N*-ethylmaleimide-sensitive factor) and additional cofactors termed SNAPs (for soluble NSF attachment proteins) (5).

Studies performed largely on recombinant proteins in solution have revealed a detailed picture of SNARE complex assembly. Of the monomeric SNAREs, only syntaxin 1 is partially  $\alpha$ -helical, whereas both SNAP-25 and synaptobrevin are largely unstructured. Upon assembly, a dramatic increase in  $\alpha$ -helical content is observed suggesting major conformational changes (6, 7). Interactions in the complex are largely confined to the SNARE motifs. X-ray crystallography revealed that the assembled core complex consists of an elongated bundle of four intertwined  $\alpha$ -helices, each representing a single SNARE motif. Two of the helices are contributed by SNAP-25, and one each by syntaxin 1 and synaptobrevin (8). The core complex is resistant to proteolysis and to denaturation by the detergent SDS (unless heated) (9). Furthermore, it is unusually heat-stable and only denatures above 80 °C (10). These and other findings suggested that SNARE complex assembly is the essential step in initiating membrane fusion. According to this hypothesis, assembly of SNAREs localized on the two opposing membranes form a tight connection between the membranes. In the resulting "trans" complexes, the SNARE motifs are partially assembled in a helical bundle, while the transmembrane domains are anchored in the still separated membranes. The energy released during assembly may, at least partially, overcome the barrier

<sup>1</sup> The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; CD, circular dichroism; DTT, dithiothreitol; FID, free induction decay; GST, glutathione *S*-transferase; HSQC, heteronuclear single quantum correlation; MALLS, multi-angle laser light scattering; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; NSF, *N*-ethylmaleimide-sensitive factor; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; SNAP-25, synaptosomal associated protein of 25 kDa; SNAP-29, synaptosomal associated protein of 29 kDa; TOCSY, total correlation spectroscopy.

separating the membranes destined to fuse. During fusion, the SNAREs relax into a complex in which all transmembrane domains are aligned in parallel ("cis" complex) and which then is re-energized by disassembly involving NSF and SNAPs (11).

In search for molecules regulating the function of neuronal SNAREs, several proteins have been identified that bind specifically to individual SNAREs or to SNARE core complexes (4). Among these are the complexins, two related proteins of approximately 15 kDa. Complexins, also named synaphins, were identified as polar and soluble proteins that are associated with the neuronal SNARE complex in membrane extracts (12–14). *In vitro*, complexins bind to assembled SNAREs and compete with  $\alpha$ -SNAP for binding (12), suggesting that they are involved in the regulation of the SNARE assembly-disassembly cycle. Both complexins are colocalized with neuronal SNAREs in presynaptic nerve terminals, although cytoplasmic pools are also present, particularly during early stages of neuronal maturation (15).

The precise function of the complexins remains to be elucidated. When the intracellular concentration of complexin was increased by microinjection or by overexpression, a moderate reduction of neurotransmitter release was observed. Injection of an anti-complexin antibody increased transmitter release, suggesting that complexins may function as negative regulators (15, 16). Disruption of the complexin II gene in mice resulted in viable mice with all parameters of synaptic transmission being normal except long term potentiation (17). However, disruption of both complexin genes causes massive neurological dysfunctions,<sup>2</sup> indicating that complexins are essential for synaptic function.

Here we have used a combination of biochemical and biophysical approaches to learn more about complexins and their interactions with SNAREs. First, we have characterized the structure of complexins. Second, we have investigated which domains of complexins are involved in binding to the SNARE complex, which structural elements of the SNAREs participate in the interaction, and how complexins influence the assembly-disassembly reactions of the SNARE proteins.

#### EXPERIMENTAL PROCEDURES

**Materials**—NSF and  $\alpha$ -SNAP in pQE-9 plasmids (Qiagen) encoding for His<sub>6</sub>-tagged fusion proteins were kindly provided by S. Whiteheart and J. E. Rothman (Memorial Sloan-Kettering Cancer Center, New York, NY). The recombinant protein fragments were derived from cDNAs encoding for rat synaptobrevin 2 and rat syntaxin 1A (kindly provided by R. H. Scheller, Stanford University School of Medicine, Stanford, CA; Refs. 18 and 19).

**Recombinant Proteins**—The pET-28a and pET-15b vector (Novagen) encoding for thrombin cleavable amino-terminal His<sub>6</sub>-tagged fusion proteins were used for the expression of the following constructs: rat synaptobrevin 2 (residues 1–116), the cytoplasmic region of synaptobrevin 2 (residues 1–96), the cytoplasmic domain of rat syntaxin 1A (residues 1–262), the SNARE complex forming part of syntaxin 1A (residues 180–262), syntaxin 1A (183–288), full-length rat SNAP-25A, SNAP-25 (1–83), and SNAP-25 (120–206). In full-length SNAP-25A, all cysteines were replaced with serines as described earlier (10, 20, 21). pHO-2 vectors encoding for COOH-terminal His<sub>6</sub>-tagged fusion proteins (7) were used for rat syntaxin 1A (1–265), syntaxin 2 (1–265), syntaxin 3 (1–260), syntaxin 4 (1–273), and rat SNAP-25A (1–206) (7, 21). Rat proteins in pGEX-KG, pGEX-1, and pGEX-2T expression vector (Amersham Pharmacia Biotech) encoding for thrombin-cleavable GST fusion proteins were as follows: GST-syntaxin 1A (1–265), GST-syntaxin 1A (1–264), GST-syntaxin 1A (180–264), GST-synaptobrevin 2 (1–94), GST-endobrevin (1–74), GST-SNAP-25A, and GST-complexins I and II (12, 21–23).

**Molecular Cloning of Recombinant Proteins**—The sequences encoding rat complexins I and II (residues 1–134), respectively, were amplified by PCR and subcloned into pET-15b vector via *Nde*I and *Xho*I restriction sites resulting in fusion proteins carrying a thrombin-cleavable amino-terminal His<sub>6</sub> tag. GST-complexin II deletion mutants (Fig.

7) were generated by PCR and subcloning into pGEX-KG vector via *Eco*RI and *Bam*HI restriction sites with a thrombin-cleavable NH<sub>2</sub>-terminal GST tag. The correct sequence of all mutants was confirmed by DNA sequencing.

SNAP-29 from rat (AF035822) was amplified by PCR using primers based on the published GS-32 sequence from rat (24). cDNA from rat lung and kidney (Stratagene) was used as template. The PCR products were subcloned into pBS vector (Stratagene) and sequenced. All constructs derived from the two different tissues were identical (GenBank<sup>TM</sup> accession no. AF260577). However, comparison with the published GS-32 sequence revealed four nucleotide discrepancies (c109g, t–153ca, c162–, t194c) resulting in two amino acid exchanges (P37A, F65S) and two frameshifts resulting in three amino acid exchanges (G52R, P53A, S54E). Comparison of the rat sequence with human SNAP-29 (AF115436) and mouse expressed sequence tags (*e.g.* AA388177, AA270049, and AA388158) further suggests that the sequence determined here is accurate: Ala<sup>37</sup> corresponds to Ala in human SNAP-29, and Arg-Ala-Glu in positions 52–54 are identical to the corresponding residues in human SNAP-29 and in the mouse expressed sequence tags. The sequence encoding for full-length SNAP-29 from rat was subcloned into pGEX-KG via *Bam*HI and *Xho*I restriction sites, resulting in a GST fusion protein.

Chimeras of rat syntaxins were generated using the overlapping primer method by Higuchi (25) and subcloned into pET-28a, resulting in the following chimeras (see Fig. 10): Syx 1/4, syntaxin 1A (1–213)/syntaxin 4 (222–233)/syntaxin 1A (226–262); and Syx 4/1, syntaxin 4-(1–221)/syntaxin 1A-(214–225)/syntaxin 4 (234–258). Sequencing of all DNAs revealed a difference from the published sequence of syntaxin 4 in a single amino acid (T216S) (26). Human and mouse syntaxin 4 contain a serine in this position. Furthermore, Ser<sup>216</sup> is conserved between rat syntaxins 1, 2, and 3, suggesting that this discrepancy is probably due to a sequencing error.

**Protein Expression and Purification**—All recombinant proteins were expressed in *E. coli* BL21 (DE3) cells according to standard protocols with the exception of GST-complexin I for NMR studies. For this purpose cells were grown at 37 °C in M9 minimal media with 4 g/liter glucose, 1 g/liter NH<sub>4</sub>Cl, 1 mM MgSO<sub>4</sub>, and 50  $\mu$ g/ml ampicillin. Uniform <sup>15</sup>N and <sup>13</sup>C labeling was achieved using <sup>15</sup>NH<sub>4</sub>Cl and [<sup>13</sup>C<sub>6</sub>]glucose (Isotec) as the sole nitrogen and carbon sources, respectively. Cells were induced at optical density 0.6 with 0.8 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside. His<sub>6</sub>-tagged proteins were affinity-purified using Ni<sup>2+</sup>-NTA-agarose (Qiagen) and GST fusion proteins by glutathione-Sepharose (Amersham Pharmacia Biotech). GST-complexin II deletion mutants (Fig. 7) remained attached to glutathione-Sepharose. Usually the tags were cleaved by thrombin after elution from the beads. However, if used for precipitation in binding experiments (see below), tags remained attached to the proteins. Proteins containing transmembrane regions (synaptobrevin(1–116) and syntaxin(183–288)) were eluted from the matrix and dialyzed against standard buffer containing 1.5% sodium cholate. All other proteins were purified by ion exchange chromatography on Mono-Q or Mono-S columns (Amersham Pharmacia Biotech) after elution from the affinity matrix. For NMR experiments, labeled complexin I without tag was buffer exchanged into 60 mM phosphate, 2 mM DTT, 0.5 mM EDTA, pH 6.1 (9:1 H<sub>2</sub>O/D<sub>2</sub>O). For binding experiments using <sup>1</sup>H-<sup>15</sup>N HSQC spectra, syntaxin (residues 1–264 and 180–264) and the "midi core complex" (synaptobrevin (1–96), syntaxin (180–262), and SNAP-25 (1–206)) were dialyzed against the same buffer. All other purified proteins were dialyzed against standard buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EDTA).

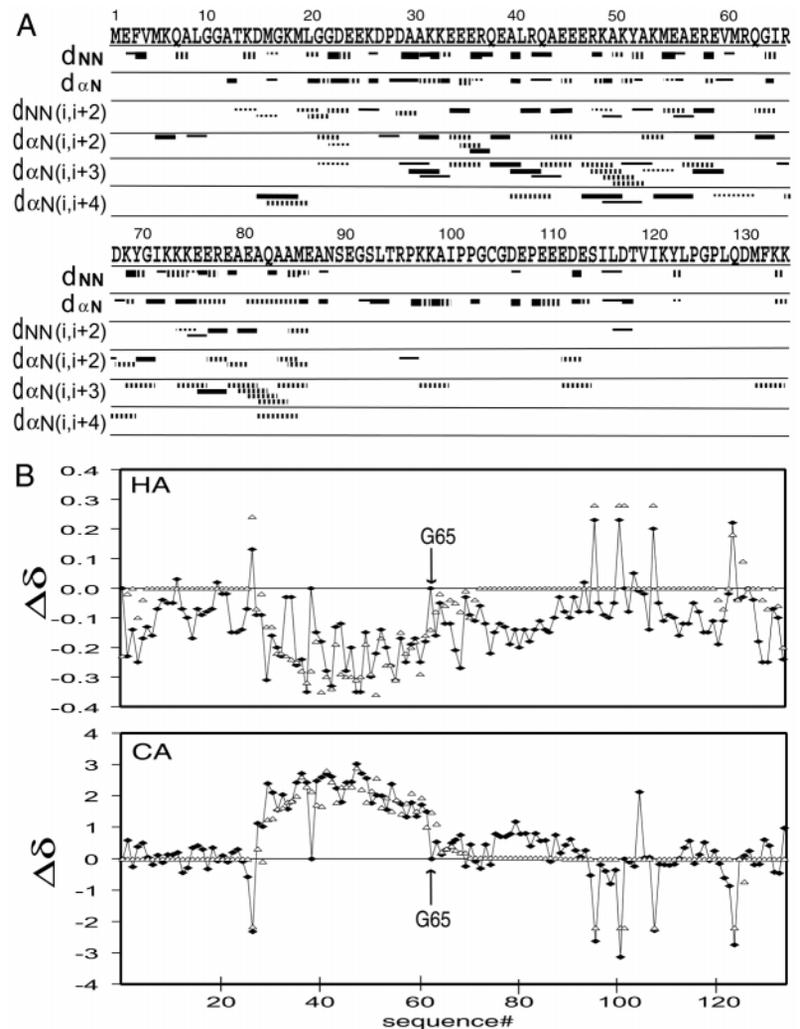
All SNARE complexes were purified using Mono-Q column after overnight assembly of the purified monomers (10, 21). For technical reasons, constructs for syntaxin 1A and SNAP-25A derived from different expression vectors were used to assemble ternary complexes: syntaxin (1–262, pET-28a), SNAP-25 (pET-28a) (Fig. 4A); syntaxin (1–265, pGEX-2T), SNAP-25 (pHO-2d) (Fig. 4B); syntaxin (1–265, pHO-2c), SNAP-25 (pHO-2d) (Fig. 5). No differences in structural and binding properties were observed. In addition, since it was not possible to separate the GST moiety from the thrombin cleaved GST-SNAP-29 fusion protein, GST-SNAP-29 was used to assemble the mixed complex containing SNAP-29. The minimal core complex consists of synaptobrevin (1–96), syntaxin (180–262), SNAP-25 (1–83), and SNAP-25 (120–206) (10). Minimal core complex with bound complexin was assembled in excess amounts of complexin II and purified by size exclusion chromatography using a Superdex 200 HiLoad 16/60 column (Amersham Pharmacia Biotech).

All protein concentrations were determined by the Bradford assay (27).

<sup>2</sup> N. Brose, personal communication.



**FIG. 3. Complexin contains a central  $\alpha$ -helix.** A, summary of sequential and medium range  $\alpha$ N and NN NOEs observed in the 3D  $^1\text{H}$ - $^{15}\text{N}$  NOESY-HSQC spectrum of  $^{15}\text{N}$ -labeled complexin I. *Solid* and *dashed lines* indicate well resolved and overlapped NOEs, respectively. The *thickness of the lines* reflects the NOE intensity. B, plots of  $\text{H}\alpha$  and  $\text{C}\alpha$  conformational shifts ( $\Delta\delta$ ) as a function of the complexin sequence. *Solid diamonds* indicate the experimental values calculated as differences between the observed chemical shifts and the random coil values described in the BioMagResBank. *Open diamonds* show the conformational shifts predicted with the program AGADIR (42), an algorithm based on the helix/coil transition theory that has been proven to be useful to predict conformational shifts and populations of secondary structure in peptides at the residue level. Note that the conformational shifts expected for an  $\alpha$ -helical conformation are negative for  $\text{H}\alpha$  and positive for  $\text{C}\alpha$ .



1.5 h at room temperature on a rotator. For immunoprecipitations, anti-synaptobrevin antibody (Cl 69.1; Ref. 31) was then added for 1 h at 4 °C. Complexes were isolated by affinity adsorption, using glutathione-Sepharose beads,  $\text{Ni}^{2+}$ -NTA-agarose, or Protein A-Sepharose (Amersham Pharmacia Biotech) for GST fusion proteins,  $\text{His}_6$  fusion proteins, and immunoprecipitations, respectively. Bead incubation was usually approximately 1 h. The beads were then washed four or five times in incubation buffer, except that Triton X-100 and bovine serum albumin were omitted and imidazole was lowered to 8 mM. Beads were analyzed by SDS-PAGE and Coomassie Blue staining. Where indicated, complexins were detected by immunoblotting using polyclonal antibody 942 (Synaptic Systems, Göttingen, Germany; 1:2000) against the COOH terminus of complexin I and II (amino acid residues 122–134, rat sequence) and enhanced chemiluminescence (ECL, NEN Life Science Products) on a Fujifilm LAS-1000 system.

**Binding to SNAREs Reconstituted in Proteoliposomes**—Proteoliposomes with reconstituted full-length synaptobrevin or ternary complex with transmembrane regions were prepared as previously described (20). Liposomes adjusted to 0.4  $\mu\text{M}$  each of synaptobrevin 2 and ternary complex, respectively, were each incubated with 0.8  $\mu\text{M}$  complexin I or II or both for 1 h at room temperature in standard buffer. Liposomes were then immunoprecipitated by incubating in monoclonal antibody Cl 69.1 (5  $\mu\text{l}$  of ascites) for 1 h at room temperature on a rotator, followed addition of Protein A-Sepharose and an additional incubation for 1 h. After sedimentation and removal of the supernatant containing unbound material, the beads were washed four times with 0.5% (v/v) Triton X-100-containing standard buffer. Fifty percent of the Sepharose-bound material were analyzed by immunoblot using antibody 942.

**Other Methods**—For limited proteolysis, purified complexes (8  $\mu\text{M}$ ) were incubated in 1% (w/w) proteinase K at 25 °C in standard buffer. At the indicated times, proteolysis was stopped by adding sample buffer without SDS containing 2 mM phenylmethylsulfonyl fluoride and chilling on ice. All samples were analyzed by nondenaturing PAGE and

Coomassie Blue staining.

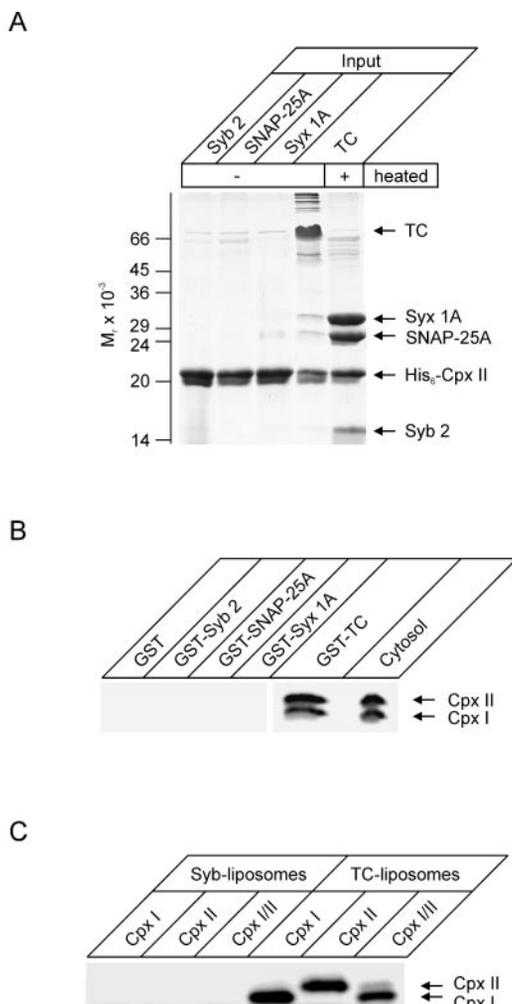
Masses of the complexin II-fragments obtained by limited proteolysis were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Perseptive). For  $\text{NH}_2$ -terminal amino acid sequencing, 1–4  $\mu\text{g}$  of complexin fragment were transferred to polyvinylidene difluoride membrane by blotting and sequenced by a Protein Sequencer 810 (Knauer).

Multi-angle laser light scattering (MALLS) and circular dichroism (CD) spectroscopy were performed as described earlier (21).

SDS-PAGE was carried out as described (32). SDS sample buffer (final concentrations: 62.5 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 3.3%  $\beta$ -mercaptoethanol) was added, and samples were incubated at room temperature (not heated) or 95 °C (heated) for 5 min before separation on 15% polyacrylamide gels. Nondenaturing gels were prepared and run as described (7).

## RESULTS

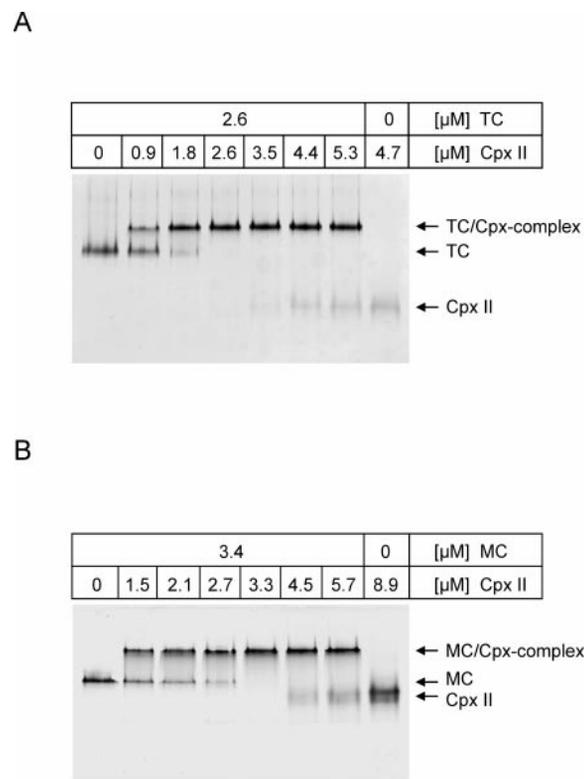
**Complexin Contains a Conserved  $\alpha$ -Helical Middle Region but Lacks a Tertiary Structure**—In vertebrates, two isoforms of complexin were described that differ only by a few amino acid substitutions and a very high degree of homology was found between distant vertebrate species. Since functionally important domains are usually more highly conserved than flanking regions, we performed data base searches to identify invertebrate orthologs of complexins, and to compare them with the vertebrate sequences. As shown in Fig. 1, sequences probably representing complexins were found both in *Drosophila melanogaster* (full-length) and *Caenorhabditis elegans* (partial). Sequence alignments revealed a high degree of overall homology between vertebrates and invertebrates, which, however, is still



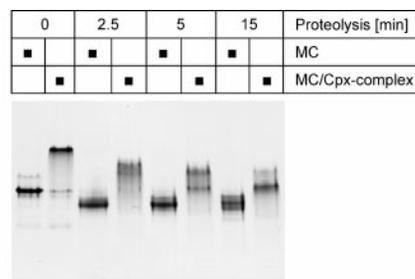
**FIG. 4. Complexins bind preferentially to the ternary SNARE complex.** *A*, purified recombinant SNAREs and ternary complex ( $4 \mu\text{M}$ ) were incubated with His<sub>6</sub>-tagged complexin II ( $3 \mu\text{M}$ ). Complexin was precipitated with Ni<sup>2+</sup>-NTA-agarose. Bound material was analyzed by SDS-PAGE and Coomassie Blue staining. In contrast to the standard protocol, the incubation buffer contained 1 mM phenylmethylsulfonyl fluoride, and DTT was omitted from the washing buffer. *B*, purified recombinant SNAREs (containing GST tags) and ternary complex (containing GST-syntaxin,  $0.75 \mu\text{M}$  each) were incubated with rat brain cytosol ( $2 \text{ mg/ml}$ , as a source of native complexins) followed by precipitation with glutathione-Sepharose. Bound complexes were analyzed by SDS-PAGE and immunoblotting. As a control,  $40 \mu\text{g}$  of cytosol were loaded. *C*, binding of recombinant complexins to proteoliposomes that were reconstituted with either synaptobrevin or ternary complex. Bound complexes were detected by immunoblot. Note that, in the presence of equal amounts of both complexins, preferential binding of complexin I is observed (*right lane*). Additional immunodetection with Cl 69.1 revealed that equal amounts of synaptobrevin and ternary complex were precipitated (data not shown). *Syb*, synaptobrevin; *Syx*, syntaxin; *TC*, ternary complex.

significantly lower than among vertebrates. The highest degree of conservation was detected in a region at the middle of the sequence spanning approximately residues 34–77 (numbering according to the rat sequence).

The structure of purified recombinant rat complexin I in solution was analyzed using nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. The <sup>1</sup>H-<sup>15</sup>N heteronuclear single quantum correlation (HSQC) spectrum of complexin (Fig. 2) exhibits a poor dispersion of <sup>1</sup>H chemical shifts that suggests a lack of tertiary structure. In contrast, the CD spectrum of complexin contains a double minimum at 206 and 222 nm (data not shown), and is characteristic for a mixture of  $\alpha$ -helical and random coil conformations. To locate the



**FIG. 5. Complexin forms a 1:1 complex with the ternary SNARE complex and with its minimal core.** The various complexes were separated by nondenaturing PAGE from each other and from free complexin. Increasing amounts of complexin II (concentrations as indicated) were added to a constant amount of ternary complex (*A*) or of the minimal core complex (*B*) and incubated in standard buffer at  $4^\circ\text{C}$  overnight or at room temperature for 1 h, respectively. Proteins were visualized by Coomassie Blue staining. *MC*, minimal core complex.



**FIG. 6. Limited proteolysis of the minimal core complex with bound complexin.** The major complexin fragments generated during proteolysis remain bound to the minimal core complex. Digests of complexin II bound to the minimal core complex were shifted in comparison to parallel digests of the minimal core complex when analyzed by nondenaturing PAGE and Coomassie Blue staining.

$\alpha$ -helical region, we used multidimensional NMR techniques. The severe overlap of the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum hindered assignment of the complexin <sup>1</sup>H, <sup>15</sup>N, and <sup>13</sup>C resonances. A combination of triple resonance spectra and three-dimensional <sup>15</sup>N-edited TOCSY-HSQC and NOESY-HSQC experiments allowed us to obtain nearly complete (97.1%) sequential assignments (Fig. 2).

The observation of numerous sequential NH/NH and medium range nuclear Overhauser effects (NOEs) (Fig. 3A) confirmed that a substantial part of the complexin sequence forms an  $\alpha$ -helical structure. The medium range NOEs are most abundant between residues 29 and 86, indicating that the

$\alpha$ -helical content is highest in this region. This conclusion is corroborated and refined by plots of  $H\alpha$  and  $C\alpha$  conformational shifts *versus* the amino acid sequence of complexin (Fig. 3B, *solid diamonds*). These plots show that a stable  $\alpha$ -helix is formed from residues 29–64, whereas residues 65–86 contain a substantial but lower population of  $\alpha$ -helix. The  $\alpha$ -helical region overlaps with the conserved middle region identified by sequence comparison (Fig. 1). Other regions of the molecule may sample helical conformations only occasionally. Overall, the NMR results indicate that complexin is 35–40%  $\alpha$ -helical.

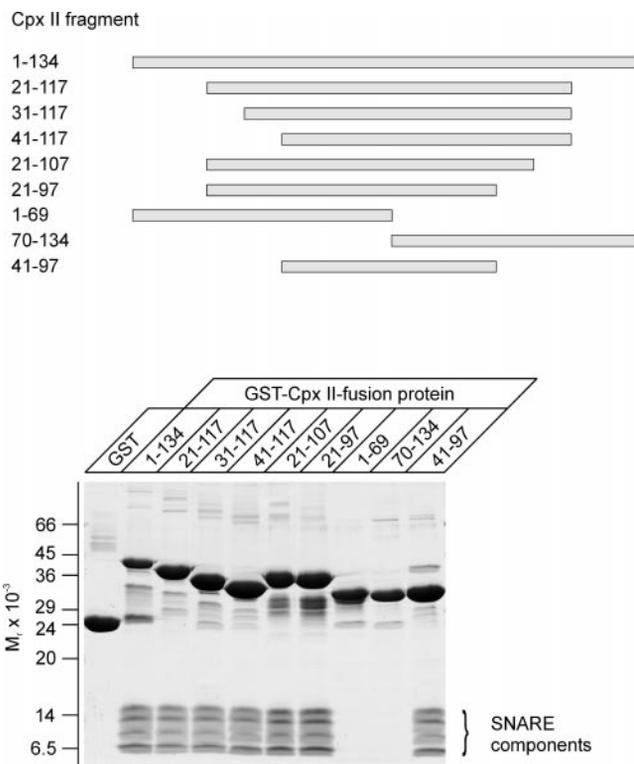
A detailed analysis of all NOE data failed to reveal any long range NOEs, confirming the absence of tertiary structure. The presence of a stable  $\alpha$ -helix in residues 29–64 is surprising since formation of stable secondary structure in the absence of tertiary interactions is unusual. However, the presence of an  $\alpha$ -helix in this region agrees with theoretical predictions (Fig. 3B). Furthermore, the  $\alpha$ -helix is not stabilized by oligomerization. Complexin is monomeric when analyzed by size exclusion chromatography combined with MALLS (data not shown); furthermore, the  $^1H$ - $^{15}N$  HSQC spectrum does not change over a wide concentration range (0.01–2 mM).

**The  $\alpha$ -Helical Middle Region of Complexin Stoichiometrically Binds to the SNARE Core Complex**—Next we investigated the binding of complexin to individual SNAREs and SNARE complexes using purified recombinant proteins and protein fragments. Unless stated otherwise, all SNAREs were expressed in bacteria without their transmembrane region. First, the binding of syntaxin 1, SNAP-25, synaptobrevin, and of preassembled ternary SNARE complex to His<sub>6</sub>-tagged complexin II was investigated. Only the ternary complex associated with complexin in significant amounts under our experimental conditions (Fig. 4A). A similar result was obtained when GST-tagged SNAREs and GST-tagged ternary complex were incubated with rat brain cytosol as a source of native complexins (Fig. 4B). Both complexin I and complexin II bound to the ternary complex, whereas no binding was observed to monomeric SNAREs. Together, these results agree with previous findings, which showed that complexins preferentially bind to assembled SNARE complexes (12).

To confirm that complexin also binds to the ternary complex when it is incorporated into a membrane, purified synaptobrevin and ternary complex (including transmembrane regions) were reconstituted into proteoliposomes by detergent removal. Binding of both complexin I and complexin II was determined by immunoprecipitation of the proteoliposomes with the aid of an anti-synaptobrevin monoclonal antibody. As shown in Fig. 4C, liposomes containing the ternary complex bound each of the complexins while no binding was observed to synaptobrevin-containing liposomes. When equal amounts of both complexin isoforms were included in the assay, preferential binding of complexin I was observed.

Next, we measured the stoichiometry of complexin binding. Preliminary experiments revealed that the association between complexin and the ternary complex is sufficiently stable to allow for its separation from both free ternary complex and the uncomplexed proteins by means of nondenaturing PAGE. Increasing concentrations of complexin II were incubated with a fixed concentration of purified ternary complex. As shown in Fig. 5A, a gradual shift from free ternary complex to complexin-bound ternary complex was observed until all free ternary complex disappeared. When more complexin was added, no further binding was observed, and unbound complexin accumulated. At equal concentrations of complexin and ternary complex, only bound complexin was detectable. We conclude that the stoichiometry between complexin and the ternary complex is 1:1.

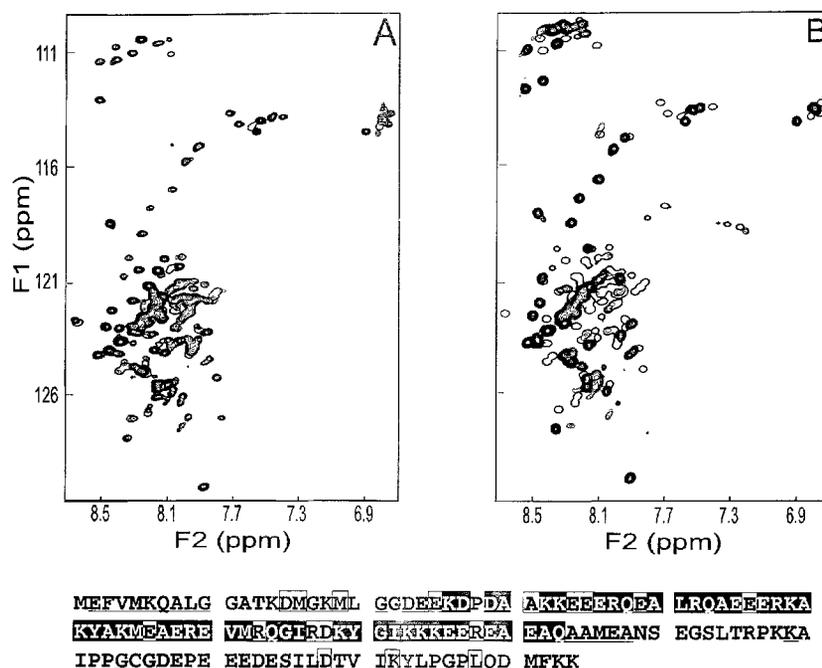
As discussed in the Introduction, assembly of SNAREs into



**FIG. 7. Mapping of the binding domain of complexin by deletion mutagenesis.** A series of truncated GST-complexin II fusion proteins was immobilized to glutathione-Sepharose beads (approximately 8  $\mu$ M each) and incubated with purified minimal core complex (13  $\mu$ M). After washing of the beads, bead-bound material was analyzed by SDS-PAGE and Coomassie Blue staining. All samples were boiled prior to separation to dissociate the SNARE components of the minimal core complex. Note that all complexin fragments bound to the minimal core complex except for fragments 1–69 and 70–134.

core complexes is confined to a region which encompasses the SNARE motifs. We have shown previously that a minimal core complex can be formed from recombinant fragments that has similar properties to the non-truncated complex with respect to assembly, disassembly, and stability (10). Since complexin binds to the ternary complex with strong preference over the individual proteins, we examined whether binding is confined to the minimal core complex. Minimal core complex was generated and purified as described previously (10), and complexin binding was analyzed as described above. As shown in Fig. 5B, complexin bound to this minimal complex with similar efficiency and with a 1:1 stoichiometry.

The stability of complexin binding to the minimal core prompted us to purify this complex for further structural investigations. After purification, the complex was subjected to time-dependent proteolysis using proteinase K, a protease with broad substrate specificity, followed by analysis with non-denaturing PAGE. As shown in Fig. 6, a gradual shift of the complex to higher mobilities was observed. Since the minimal core is largely resistant to proteolysis (Fig. 6; see also Refs. 10 and 33), this shift is mostly due to truncation of complexin. SDS-PAGE revealed several complexin fragments that were further analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and by  $NH_2$ -terminal sequencing. Proteolysis was first observed at the  $NH_2$  terminus, resulting in a peptide containing residues 21–134. When the incubation time was extended, progressive COOH-terminal truncations were observed (including fragments containing residues 21–116), while no further shortening of the  $NH_2$  terminus was detected.



**FIG. 8. Mapping of the binding domain of complexin by NMR: complexin binds to the core complex via its central  $\alpha$ -helix.**  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of isolated  $^{15}\text{N}$ -complexin I (single thick contours) superimposed with  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra acquired on identical samples after addition of one equivalent of syntaxin SNARE motif (residues 180–264) (A) or midi core complex (B) (single thin contours). The experiments were acquired at 500 MHz (A) or 600 MHz (B) under the conditions described in Fig. 2, except that the concentration of all proteins was 90  $\mu\text{M}$  (A) or 40  $\mu\text{M}$  (B). Note that some cross-peaks from NH groups in fast exchange with the solvent are observed only at 600 MHz (e.g. those from Arg side chains on the right side of the spectrum). At the bottom, the sequence of complexin I is shown summarizing the spectral changes observed upon addition of the midi core complex (spectra shown in B). Boxed residues with a black background correspond to cross-peaks that broaden beyond detection, and underlined residues correspond to cross-peaks with moderate broadening or slight shifts. For some cross-peaks, spectral changes cannot be assessed due to severe overlap and the corresponding residues are indicated by open boxes. All experiments were performed at protein concentrations below 100  $\mu\text{M}$  to minimize aggregation.

To further define the complexin binding region, we generated a series of deletion mutants of complexin II using the proteolysis experiment as a guide. As shown in Fig. 7, progressive deletion at both COOH- and  $\text{NH}_2$ -terminal ends did not abolish binding, with the smallest binding fragment encompassing residues 41–97. In contrast, no binding was observed when the protein was “cut” in the middle, *i.e.* with the COOH- and  $\text{NH}_2$ -terminal halves of complexin. In addition, no binding was observed when both fragments were added together (data not shown).

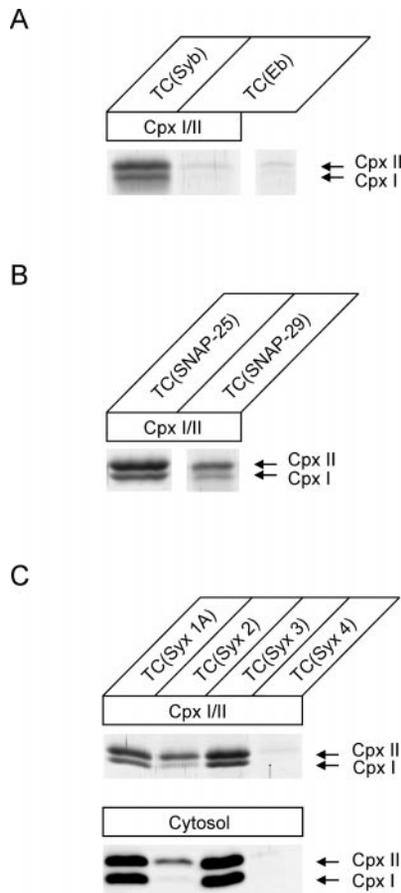
The data described so far suggest that the binding region of complexin corresponds approximately to the conserved  $\alpha$ -helical region characterized above. To confirm and extend these findings, we used NMR spectroscopy. The changes in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of a  $^{15}\text{N}$ -labeled protein caused by binding to an unlabeled protein can be used to map the region of the labeled protein involved in the interaction (34). For a  $^{15}\text{N}$ -labeled protein lacking a tertiary structure such as complexin, it is expected that interaction with another protein will result in severe broadening of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC cross-peaks from the region involved in binding due to the resulting increase in correlation time. Regions not involved in binding are expected to retain internal motions, and their  $^1\text{H}$ - $^{15}\text{N}$  HSQC cross-peaks should remain largely unaffected (35).

We first recorded  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$ -labeled complexin I in the absence and presence of unlabeled cytoplasmic region of syntaxin 1A (residues 1–264). No spectral changes were observed in these experiments (data not shown), in good agreement with the biochemical observations. Since binding could be hindered by the intramolecular interaction of the  $\text{NH}_2$ -terminal domain of syntaxin with its COOH-terminal SNARE motif (23), we performed additional  $^1\text{H}$ - $^{15}\text{N}$  HSQC experiments using  $^{15}\text{N}$ -labeled complexin and an unlabeled

fragment of syntaxin (residues 180–264) containing only the SNARE motif. Addition of this fragment caused broadening of a few  $^1\text{H}$ - $^{15}\text{N}$  HSQC cross-peaks from complexin (Fig. 8A). However, the residues corresponding to these cross-peaks are not clustered in any specific region but instead are spread throughout the complexin sequence. These results indicate that the observed interaction is not specific.

Next we acquired  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$ -labeled complexin in the presence of unlabeled ternary complex containing a  $\text{NH}_2$ -terminally truncated version of syntaxin 1A (midi core complex, syntaxin residues 180–262). About half of the  $^1\text{H}$ - $^{15}\text{N}$  HSQC cross-peaks of complexin were broadened beyond detection, and several cross-peaks exhibited partial broadening and/or slight shifts (Fig. 8B). The spectral changes are summarized on the sequence of complexin at the bottom of Fig. 8. All cross-peaks that broadened beyond detection correspond to the region encompassing residues 26–83, which approximately coincides with the helical region at the center of the complexin sequence. Furthermore, moderate perturbations (broadening and/or shifts) were observed in the  $\text{NH}_2$ -terminal region (residues 1–25) and the very COOH terminus (residues 121–134). These perturbations most likely arise from nonspecific binding modes. However, it is also possible that the  $\text{NH}_2$ - and COOH-terminal regions of complexin may be involved in direct or indirect interactions with the core complex in the presence of additional components of the exocytotic machinery.

**Complexin Binding Is Sensitive to Exchange of Syntaxin and Synaptobrevin**—As discussed above, the core domain of the SNARE complex consists of a bundle of four twisted  $\alpha$ -helices that are connected by leucine zipper-like layers of interacting hydrophobic amino acids in the center of the bundle. Mapping SNARE sequences on the crystal structure revealed that the interacting amino acids are more conserved than the residues



**FIG. 9. Substitution of syntaxin 1 and synaptobrevin 2 but not of SNAP-25 abolishes binding of complexins to ternary SNARE complexes.** The following substitutions were carried out: endobrevin (*Eb*) for synaptobrevin 2 (*A*), SNAP-29 for SNAP-25 (*B*), and syntaxins 2–4 for syntaxin 1A (*C*). In each of the SNARE complexes at least one of the proteins carried a His<sub>6</sub> tag. The complexes (1.5  $\mu$ M each) were incubated with recombinant complexins (2.0  $\mu$ M in *A* and *B*, 1.5  $\mu$ M in *C*). In *panel C* (bottom), rat brain cytosol (2 mg/ml) was used instead. After isolation by Ni<sup>2+</sup>-NTA-agarose beads, bound complexins were detected by SDS-PAGE followed by Coomassie Blue staining (*A*, *B*, and *C* top panel) or immunoblotting (*C*, bottom panel). Note that no binding is observed in complexes containing endobrevin or syntaxin 4. For *A* and *B*, incubation buffer with 1 mM phenylmethylsulfonyl fluoride was used (see standard protocol). For *C* (top panel), incubation buffer contained 120 mM NaCl, but no DTT. Incubation with native complexins (*C*, bottom panel) was carried out at 4 °C for 2 h. For all experiments, washing buffer without DTT was used and samples were heated prior to electrophoresis.

exposed on the surface (3), explaining why SNARE complexes form promiscuously between different SNARE proteins (21, 36). However, substitution of one SNARE for another will usually result in a profound alteration of the surface pattern, which thus distinguishes different SNARE complexes from each other. Since complexin binds to the surface of the assembled core domain and most likely does not have access to its hydrophobic core, it is conceivable that complexin binding is more sensitive to SNARE substitution than SNARE complex formation.

To examine whether substitution of SNAREs affects binding of complexins, we generated mixed SNARE complexes in which one of the neuronal SNAREs was substituted by a different family member. All mixed complexes were purified, are stable, and can be disassembled by the ATPase NSF (21, 36). As shown in Fig. 9A, replacement of synaptobrevin with the endosomal relative endobrevin abolished binding of complexins. In contrast, binding was largely preserved when SNAP-25 was re-

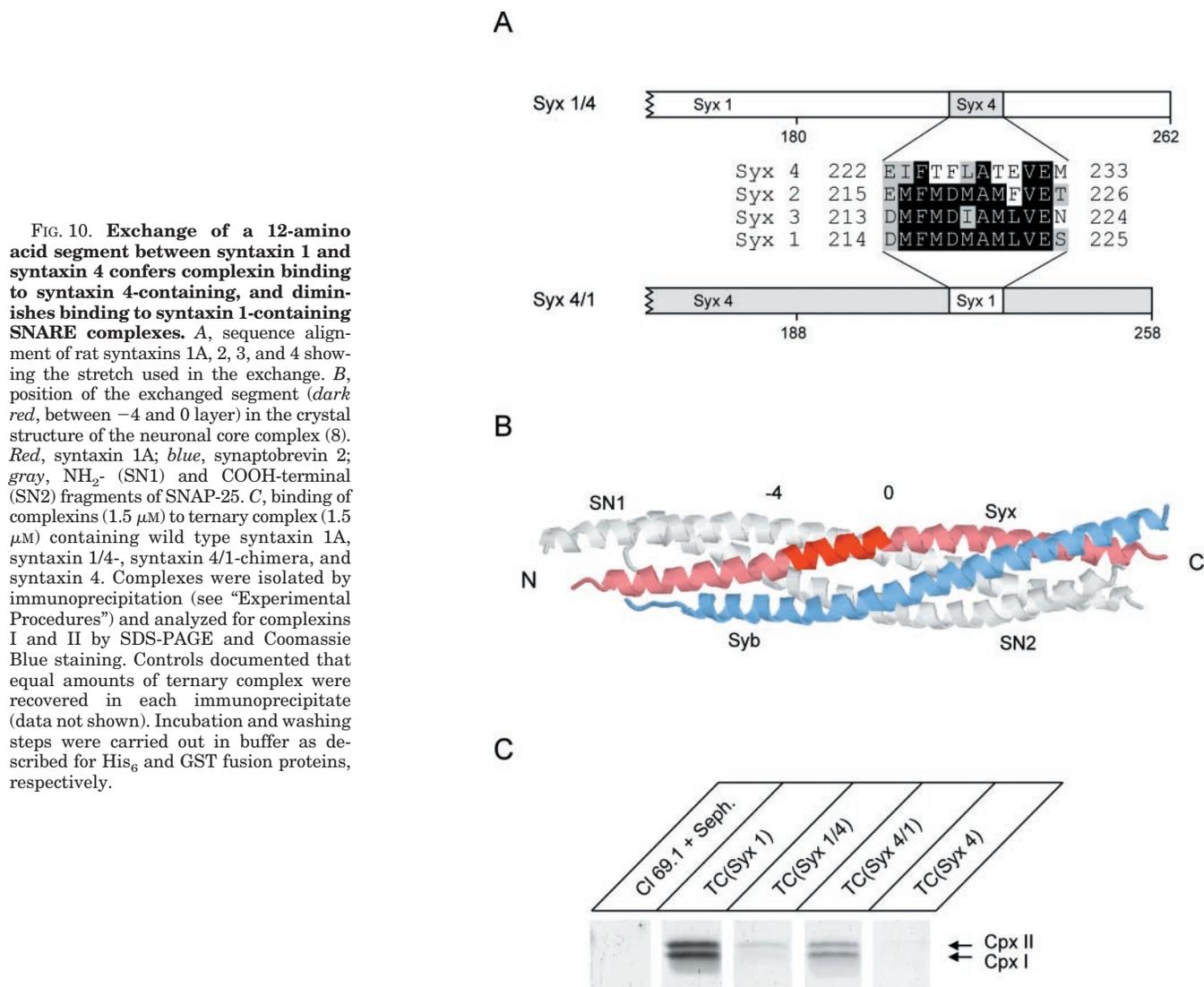
placed by its distant relative SNAP-29 (Fig. 9B). Since SNAP-25 and SNAP-29 are only 19.9% similar to each other (Clustal method with PAM250 residue weight table), and the conserved residues are mostly confined to the hydrophobic core of the SNARE complex, this result indicates that the SNAP-25 side chains contribute less to complexin binding. Finally, we replaced syntaxin 1 with syntaxins 2, 3, and 4. Complexins bound with equal efficiency to the syntaxin 3-containing complexes (Fig. 9C), which agrees with previous observations suggesting an association of complexin with syntaxin 3, synaptobrevin, and SNAP-25 in the retina (37). Replacement by syntaxin 2 reduced binding, with a preference for complexin II, whereas replacement by syntaxin 4 abolished binding.

In contrast to synaptobrevin/endobrevin and SNAP-25/SNAP-29, the SNARE motifs of syntaxins 1–4 display a relatively high degree of homology. To narrow down the sequence responsible for the observed differences in complexin binding, we compared syntaxins 1 and syntaxin 4 in order to identify the regions with the lowest degree of homology between the surface-exposed residues. The most conspicuous stretch includes residues 214–225 (numbering of syntaxin 1). To test whether this stretch is involved in complexin binding, we generated chimeric proteins by swapping these stretches of 12 residues between syntaxin 1 and syntaxin 4. The chimeras were then used to form ternary complexes with synaptobrevin and SNAP-25 that were purified and used in complexin binding assays. As shown in Fig. 10, the complex formed with the chimera of syntaxin 1 containing the stretch of syntaxin 4 bound significantly less complexins than the unmodified complex. Conversely, the syntaxin 4 chimera containing the stretch of syntaxin 1 exhibited partially restored complexin binding. These findings show that this stretch plays a key role in defining the binding site for complexins, although it is most probable that flanking regions of the molecule are also involved.

**Complexin Does Not Affect Disassembly of the SNARE Complex**—In the final series of experiments, we investigated whether complexin has an influence on the disassembly of preformed SNARE complexes by NSF and  $\alpha$ -SNAP. The minimal core of the neuronal SNARE complex was purified either with or without bound complexin II. For disassembly, purified  $\alpha$ -SNAP and NSF were added in the presence of ATP. Since the minimal core complex is stable during SDS-PAGE, the appearance of the monomeric SNARE components is indicative of disassembly. As shown in Fig. 11A, disassembly was observed independently of whether complexin was bound or not. The minimal core complex was also disassembled when complexin was added in a 20-fold excess over  $\alpha$ -SNAP, even when the incubation time was reduced to 5 min (Fig. 11B). Furthermore, no influence of complexin on disassembly was observed when the non-truncated versions of the SNARE proteins were used (data not shown). We also performed preliminary experiments on isolated synaptic vesicles to explore whether complexin has an effect on disassembly of native SNARE complexes. However, no changes were found in the presence of complexins (data not shown).

## DISCUSSION

Complexin is a small and hydrophilic protein that was identified by its ability to bind to the neuronal SNARE complex (12, 13). In this study, we have used a combination of approaches to characterize this interaction in detail. Our results show that complexin binds stoichiometrically to the ternary SNARE complex, whereas it exhibits no binding to the individual SNAREs. Free complexin lacks a tertiary structure but contains a conserved  $\alpha$ -helical domain at the center of its sequence which mediates binding. Furthermore, complexin is able to discriminate between different SNARE complexes. Substitution of in-



**FIG. 10. Exchange of a 12-amino acid segment between syntaxin 1 and syntaxin 4 confers complexin binding to syntaxin 4-containing, and diminishes binding to syntaxin 1-containing SNARE complexes.** *A*, sequence alignment of rat syntaxins 1A, 2, 3, and 4 showing the stretch used in the exchange. *B*, position of the exchanged segment (dark red, between -4 and 0 layer) in the crystal structure of the neuronal core complex (8). Red, syntaxin 1A; blue, synaptobrevin 2; gray, NH<sub>2</sub>- (SN1) and COOH-terminal (SN2) fragments of SNAP-25. *C*, binding of complexins (1.5  $\mu$ M) to ternary complex (1.5  $\mu$ M) containing wild type syntaxin 1A, syntaxin 1/4-, syntaxin 4/1-chimera, and syntaxin 4. Complexes were isolated by immunoprecipitation (see "Experimental Procedures") and analyzed for complexins I and II by SDS-PAGE and Coomassie Blue staining. Controls documented that equal amounts of ternary complex were recovered in each immunoprecipitate (data not shown). Incubation and washing steps were carried out in buffer as described for His<sub>6</sub> and GST fusion proteins, respectively.

dividual SNAREs and site-directed mutagenesis suggest that complexin binds predominantly to the groove formed by syntaxin and synaptobrevin at the surface of the core complex.

The central  $\alpha$ -helix of complexin was shown to be responsible for binding to the SNARE complex by means of three independent approaches: limited proteolysis, deletion mutagenesis, and NMR spectroscopy. Thus, the binding region of complexin corresponds to the structured part of the molecule. Furthermore, we were unable to detect signs for major conformational changes of either complexin or the core complex during the binding reaction. These features differ from the assembly of SNARE motifs that are mostly unstructured as monomers and that undergo massive structural changes upon complex formation (6, 7). The binding domain of complexin contains more than 60% charged residues. Since the surface of the neuronal core complex is also highly polar (8), it is likely that polar and electrostatic interactions are primarily responsible for binding.

Although the precise nature of complexin binding to the core complex remains to be established, several conclusions can be drawn from our results. First, the central region of the core complex is critical for complexin binding, as shown by site-directed mutagenesis of syntaxin 1 (Fig. 10). Second, the membrane-proximal region of the core complex does not appear to be involved because complexes formed from COOH-terminally truncated SNAREs (e.g. by clostridial neurotoxins (Ref. 38))

bind with comparable efficiency.<sup>3</sup> Third, syntaxin 1 and synaptobrevin are more important for complexin binding than SNAP-25. Since, in the crystal structure of the neuronal SNARE complex, the helices of syntaxin and synaptobrevin form one of the grooves extending through the length of the bundle, it is possible that the helix of complexin aligns along this groove during binding.

One of our most interesting findings shows that complexin is capable of distinguishing between core complexes formed from different sets of SNAREs. Such complexes are predicted to differ much more in their surface patterns than in their core interactions (3). Indeed, *in vitro* studies have shown that SNAREs form core complexes rather indiscriminately (21, 36), and consequently one must look elsewhere for an explanation for the intracellular specificity of SNARE interactions and their regulation. Although no other relatives of complexin have been identified so far, it is possible that other related proteins exist that bind with comparable selectivity to different SNARE complexes.

How does complexin regulate the functioning of neuronal SNAREs? Although this question cannot be answered at present, some important features are beginning to emerge. First, it is unlikely that complexin regulates pools of individual

<sup>3</sup> S. Pabst, R. Jahn, and D. Fasshauer, unpublished observations.



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**MEMBRANE TRANSPORT STRUCTURE  
FUNCTION AND BIOGENESIS:  
Selective Interaction of Complexin with the  
Neuronal SNARE Complex:  
DETERMINATION OF THE BINDING  
REGIONS**

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